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Detection of Enteroviral RNA by Polymerase Chain Reaction in Endomyocardial Tissue of Patients With Chronic Cardiac Diseases

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Enteroviruses are suspected to be etiologic agents in myocarditis and cardiomyopathy. The prevalence of enteroviral (EV) heart infection in patients with chronic cardiomyopathy was determined through detection of specific EV genomic sequences using reverse transcription and polymerase chain reaction (RT-PCR) followed by slot blotting. Endomyocardial biopsies from the explanted hearts of 19 patients with dilated cardiomyopathy (DCM) and 14 patients with chronic coronary disease (CCD) were examined. EV genome was detected in 11 of 19 patients with DCM and in 8 of 14 patients with CCD. Ventricular biopsies from the control group, which included 35 healthy heart patients and 33 patients with myocardial infarction, were negative by EV RT-PCR. The percentage of patients showing presence of EV-RNA was almost similar in the DCM (57.9%) and CCD (57.1%) groups. The present study demonstrates that enterovirus RNA sequences persist in the myocardium in a significant proportion of patients suffering from end-stage ischaemic and dilated cardiac diseases and supports the hypothesis of a possible direct link between EV infection and the pathogenesis of chronic heart disease.

KEY WORDS: enteroviruses, chronic cardiomyopathy, RT-PCR, heart transplantation

INTRODUCTION

Human enteroviruses are implicated in numerous diseases and are widespread in the world. Among these viruses, Coxsackieviruses B (CVB; B1–B6 serotypes) are suspected to be etiologic agents in chronic forms of myocarditis [Schwaiger et al., 1993; Kandolf et al., 1993]. Evidence for enteroviral involvement in acute myocarditis was suggested by the detection of CVB-specific neutralizing IgM and IgG antibodies [Woodruff, 1980]. The presence in patients' sera of CVB3-specific IgM after a phase of myocarditis indicated that EVs could also establish chronic infection that would result in a chronic heart syndrome, dilated cardiomyopathy (DCM) [Muir et al., 1989]. At this stage, the finding of enteroviral particles or viral antigens in myocardium using conventional biological techniques was unsuccessful [Woodruff, 1980].

Recently, new enteroviral genomic sequence and genetic data allowed the development of specific entoviral (EV) genomic detection [Rotbart, 1990a]. Numerous hybridization studies using slot blot or in situ hybridization demonstrated positive EV detection in myocardium from patients with DCM but provided conflicting results regarding prevalence, which ranged from 15% to 42% [Kandolf et al., 1987; Kandolf, 1988; Archard et al., 1993]. Recently, the use of reverse transcription-polymerase chain reaction (RT-PCR) has allowed a more specific and sensitive EV detection in human myocardium [Rotbart, 1990a]. The presence of EV RNA was shown in explanted hearts from a variable number of patients (0–66%) undergoing transplantation due to end-stage disease, but also from control patients with other cardiac diseases [Petitjean et al., 1992; Keeling et al., 1992; Liljeqvist et al., 1993]. However, the lack of discrimination between test and control groups, the absence of healthy heart patients groups, and the controversial specificity of EV primers and molecular probes used prevented establishment of a clear link between persistent EV heart infection and DCM pathogenesis [Petitjean et al., 1992].

In the present study, the involvement of EV as etiological agent in chronic cardiomyopathy and especially in DCM was investigated by detection of EV RNA in myocardium from patients suffering from end-stage chronic cardiomyopathy vs. patients suffering from myocardial infarction and healthy heart patients (control group). Our study differs from earlier studies by the

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inclusion of a large number of test and appropriate control individuals (healthy heart patients) and by the use of combined detection assay of RT-PCR and slot blot hybridization. In this study, the strategy for the viral genomic amplification was based on an annealing of specific primers in a nucleic acid sequence of the 5′ noncoding region, which is highly conserved among the enteroviruses [Rotbart, 1990b].

MATERIALS AND METHODS

Transplanted Patient Group

Dilated cardiomyopathy. From September, 1993, to March, 1995, we studied 19 patients (14 males and 5 females) suffering from dilated cardiomyopathy according to the World Health Organization criteria and whose ages ranged from 27 to 59 years (mean age 44.5 years) [Bradenberg et al., 1980]. Their end-stage heart disease required cardiac transplantation. Seven were alcoholic, two were suffering from congenital cardiopathy, and ten were considered as suffering from idiopathic dilated cardiomyopathy. For each patient, ventricular endomyocardial biopsy was obtained at the time of cardiac transplantation, frozen quickly in liquid nitrogen, and stored at −80°C. For each patient, a part of the myocardial biopsy was examined for histopathology using haematoxylin-eosin staining. Histological findings have shown a focal fibrosis and the absence of inflammatory cells, which are DCM-like features [Kandolf et al., 1993].

Chronic coronary disease. We studied 14 patients (10 males and 4 females) suffering from ischaemic cardiac disease defined as a significant coronary heart disease resulting in the requirement for cardiac transplantation. The age of these patients varied from 47 to 79 years (mean age 58 years), and endomyocardial biopsies were collected according to the protocol of the DCM patient group. For each patient, a part of the endomyocardial biopsy was examined for histopathology to confirm the clinical findings of ischaemic heart disease.

Control Groups

Healthy heart patients. A first group of controls included 11 endomyocardial biopsies obtained at the time of necropsy (maximal delay 12 hr after death) from children. The age ranged from 2 months to 8 years (mean age 1.8 years). Myocardial biopsies were collected from 24 young adult organ donors who died suddenly of accidents or committed suicide. None of these subjects was suffering from any known cardiac pathology. All these control samples were taken from the ventricle and were frozen quickly in liquid nitrogen and stored at −80°C. For all the children and adult controls, the myocardial biopsies were cut and stained with haematoxylin-eosin, and none showed histological abnormalities.

Patients suffering from myocardial infarction. A second group of controls included 33 ventricular biopsies obtained during extracorporeal circulation for surgical treatment (coronary bypass) in patients (24 males and 9 females) suffering from myocardial infarction. Their age ranged from 39 to 87 years (mean age 63.6 years), and they did not require heart transplantation.

Cell Culture

A part of each endomyocardial biopsy was homogenized in 1 ml MEM, and 50 μl of the samples were inoculated in duplicate into 24-well plates covered with monolayers of continuous epithelial cells (Hep2) [Chonmaitree et al., 1988]. Cell cultures infected with CVB (B1–B6), polioviruses 1–3, and echovirus 11 were used as positive control in our RT-PCR assays; cultures infected with human cytomegalovirus (HCMV), varicella-zoster virus (VZV), and herpes simplex virus (HSV) type 1 and type 2 were used as negative controls for the validation of RT-PCR assay.

RT-PCR Procedure

RNA extraction. Native RNA was extracted with RNAzole (Bioprobe Systems, Montreuil Sous Bois, France) from myocardial biopsy specimens according to the one-step procedure of Chomczynski and Sacchi [1987]. Cardiac heart samples were homogenized in RNAzole (guanidium/phenol; Bioprobe Systems) and chloroform (10% v/v). Extracted RNA was then precipitated with isopropanol at −20°C overnight. After centrifugation (12,000*g* for 15 min), the pellet was washed twice in 75% ethanol and vacuum dried. The RNA was then dissolved in 50 μl diethylpyrocarbonate-treated water and used in the RT-PCR assays.

RT-PCR amplification. Two oligonucleotides (primer HPX001: 5′-AAG CAC TTC TGT TTC C-3′; primer HPX002: 5′-CAT TCA GGG GCC GGA GGA-3′) were selected for annealing in the 5′ noncoding region, which is highly conserved among the enterovirus group. These primers flank a 297 bp RNA sequence amplified in our RT-PCR assays. A 5′ biotinylated oligonucleotic probe (5′-GGC CGC CAA CGC AGC C-3′; was then applied to identify the amplified product Genset, Paris, France).

First strand complementary desoxyribonucleic acid (cDNA) was synthesised in a total volume of 25 μl using 5 μl sample material, 40 IU reverse transcriptase (M-MULV; Boehringer, Mannheim, Germany), 40 IU RNAsin (Boehringer), 5 μl reaction buffer 5× (250 mM Tris-HCl, pH 8.3; 15 mM $MgCl_2$; 350 mM KCl; 50 mM dithiothreitol; Boehringer), 0.3 μl of a solution containing 25 mM of each nucleoside triphosphate (Boehringer), 25 pmol primer HPX002, and 9.2 μl sterile water. The reaction was carried out at 37°C for 60 min and was stopped by heating the samples for 5 min at 95°C. Seventy five microliters of PCR mixture consisting of 10 μl 10× reaction buffer (100 mM Tris-HCl, pH 8.3, at 20°C; 15 mM $MgCl_2$; 500 mM KCl; gelatin: 1 mg/ml), 2.5 IU Taq-DNA polymerase (Boehringer), 25 pmol upstream and downstream primers, 0.8 μl of a solution containing 25 mM of each nucleoside triphosphate (Boehringer), and 43.7 μl sterile water was added to the cDNA sample. DNA amplification was carried out in a Perkin Elmer thermocycler 480 (Perkin Elmer, Nor-

walk, CT) for 35 cycles (denaturation 94°C, 2 min; annealing 55°C, 2 min; extension 72°C; 2 min). Each test included a positive control (total RNA extracted from EV-infected Hep2 cell culture) and a negative control (sterile water mixed with PCR reagents) [Rotbart, 1990b].

For all myocardial specimens, the glyceraldehyde phosphate dehydrogenase (G3PDH) mRNA was amplified using specific primers (P1: TGA AGG TCC GAG CAA CGG ATT TGGT-3′; P2: 5′-CAT GTG GGC CAT TTG AGG TCC CAC CAC-3′; Clontech, Palo Alto, CA, USA) in a RT-PCR and used as a positive control to prove the absence of Taq-DNA polymerase inhibitors and, to verify the RNA extraction and adequate transcription of cDNA [Schwaiger et al., 1993].

Analysis of PCR products. An aliquot of amplified RT-PCR product (25 μl) was subjected to electrophoresis at 100 V in 1.6% agarose gels containing 0.5 μl/ml ethidium bromide (Sigma, St Louis, MO). A 100 bp DNA ladder (GIBCO BRL, Eragny, France) was used as molecular weight marker. The specificity of the RT-PCR products was verified by undertaking slot blot hybridization only nylon membranes using a specific biotinylated desoxyribonucleic probe. Another aliquot of amplified product (50 μl) was diluted (v/v) in distilled water, denatured at 95°C for 10 min, cooled at 0°C for 15 min, and blotted onto a nylon membrane (Biohylon Z+ bioprobe; Bioprobe Systems) in a slot blot apparatus (PR 600; Hoeffer, San Francisco, CA). After three washings with SSC 2× buffer (1×: 15 mM sodium citrate, 150 mM NaCl), the DNA was then fixed with filter paper soaked in 0.4 M NaOH; the nylon membrane was air dried and prehybridized at room temperature for 30 min in a solution (100 μl/cm^2) containing 4× Denhardt's solution, 2.5% dextran sulfate, 10 mM Tris-HCl, pH 7.5; at 20°C; 0.1% SDS; 2% skim milk. The membrane was then transferred into the hybridization solution (100 μl/cm^2) containing the 5′ biotinylated probe (300 ng/ml), and the molecular hybridization was carried out at 40°C for 2 hr. The blot was washed twice in SSC 1× buffer with 0.1% SDS for 10 min and twice in SSC 0.1× buffer for 5 min. An alkaline phosphatase-labelled steptavidin (Dako NS, Copenhagen, Denmark) was then used at room temperature for 30 min to detect the specific hybridization of the biotinylated probe. After two washing phases with SSC 1× buffer and SSC 0.1× buffer, the labelled hybrids were detected by an enzyme color assay with chromogen P.A+ (100 μl/cm^2; Bioprobe) for 2 hr, followed by washing in distilled water to stop the reaction.

RESULTS

Validity of EV-RNA Detection Assay

Extracted RNA from polioviruses 1–3, echovirus 11, and Coxsackieviruses B1–6-infected cells gave positive results on RT-PCR in agarose gel (297 bp band) and by slot blot hybridization, demonstrating the reliability of the set of primers chosen. The specificity of RT-PCR was confirmed by the absence of amplification products with RNA extracted from Herpesvirus family-infected and noninfected Hep2 cells (data not shown). The sensitivity of RT-PCR assay was assessed by limit detection of the signal in serial tenfold dilutions of CVB3 virus added to EV-negative biopsy material and showed after gel agarose electrophoresis that the assay was able to detect RNA from 10 pfu/mg cardiac tissue according to the protocol described by Severini et al. [1993] (Fig. 1A). However, the detection of the amplification products by slot blot hybridization gave a tenfold higher sensitivity than electrophoresis (Fig. 1B). For each endomyocardial biopsy (n = 101), the G3PDH mRNA was coamplified as an internal control to verify removal of PCR inhibitors from RNA extract, which was essential for a sensitive PCR test of clinical specimens [Hierholzer et al., 1993] (Fig. 2A).

Enteroviral RNA Detection in Human Cardiac Tissue

Within the first control group (healthy heart subjects), none of the 11 myocardial biopsies collected from children and none of the 24 biopsies obtained from adult organ donors was RT-PCR positive nor cell culture positive. Within the second control group of patients suffering from myocardial infarction, enteroviral RT-PCR and cell culture detection were negative in the 33 biopsies tested (Table I).

As was reported previously, the sensitivity of the EV gene amplification methodology was increased by a molecular probe hybridization [Weiss et al., 1991; Schwaiger et al., 1993]. Indeed 47% of the 33 cardiac transplant recipients studied (DCM and CCD groups) were RT-PCR positive when analysed by gel agarose electrophoresis, whereas 57.6% were positive when analysed further by slot blotting (data not shown).

In the DCM group, enteroviral nucleic acid sequences were found in 11 (57.9%) of 19 patients (Table I). Figure 2A and B show examples of detection by agarose gel electrophoresis after RT-PCR and by slot blotting, respectively. Among the 11 positive patients of the DCM group, eight were considered as suffering from idiopathic dilated cardiomyopathy. Moreover, the prevalence of enteroviral genomic detection is highly significant in the idiopathic dilated cardiomyopathy subgroup (80%; Table 1). However, to assess whether infectious enteroviral particles could persist in the cardiac tissue at the end stage of DCM, 100 mg cardiac tissue was homogenized and inoculated into Hep2 cell culture. None of the DCM group biopsies was culture positive 14 days after inoculation.

In the group of patients suffering from CCD, 8 of 14 patients (57.1%) were positive for enteroviral RNA by RT-PCR (Table I), but the detection of infectious enteroviral particles in heart tissue was negative when inoculated into Hep2 cells in culture. Moreover, the statistical analysis of the RT-PCR data demonstrated that there was no significant difference in the prevalence of enteroviral infection between CCD (57.1%) and DCM (57.9%) patients ($P > 0.5$; Table I).

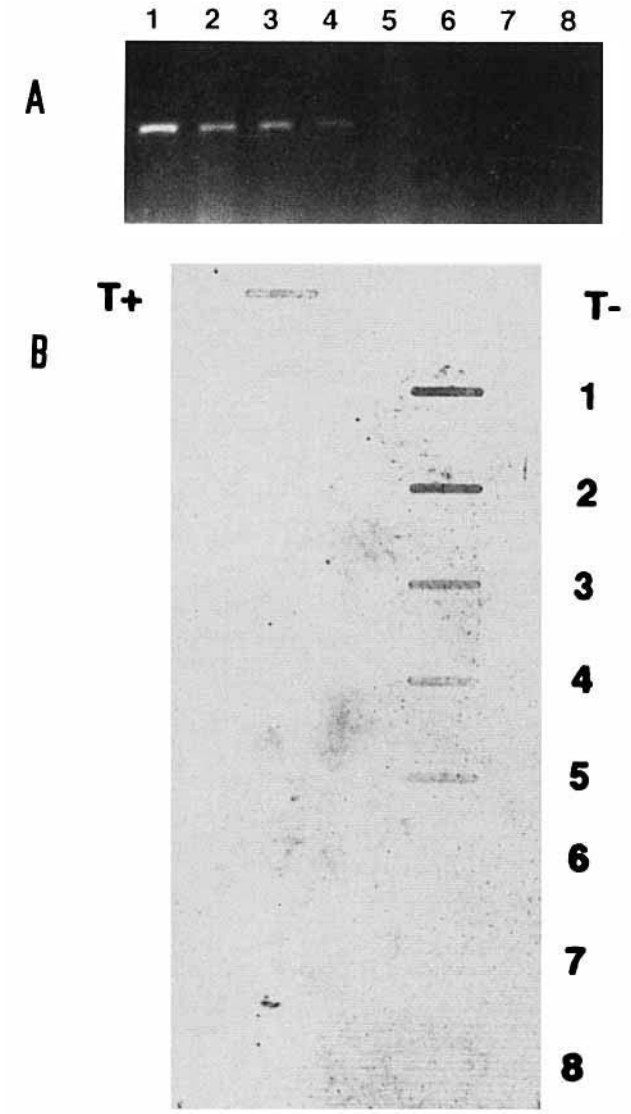

Fig. 1. **A:** Sensitivity of agarose gel electrophoresis detection of enteroviral RT-PCR products. Total RNA was extracted and subjected to RT-PCR amplification from serial tenfold dilutions of the amount of virus mixed with 1 mg myocardial tissue. **Lane 1** corresponds to 10^4 pfu of Coxsackievirus B3 per milligram of cardiac tissue and **lane 8** to 10^{-3} pfu of Coxsackievirus B3 per milligram of cardiac tissue. A 297 pb band was shown for **lanes 1–4,** which represents sensitivity of 10 pfu/mg human myocardial tissue. A negative detection was shown for **lanes 5–7** which corresponds to 1, 10^{-1}, and 10^{-2} pfu, respectively, of Coxsackievirus B3 per milligram of cardiac tissue. **B:** Sensitivity of slot blotting revelation of enteroviral RT-PCR products presented in A. **Slot 1** corresponds to the detection of 10^4 pfu Coxsackievirus B3 per milligram of cardiac tissue and slot 8 to 10^{-3} pfu of Coxsackievirus B3 per milligram of cardiac tissue. A positive signal was shown for **slots 1–5** corresponding to a sensitivity of 1 pfu/mg human cardiac tissue. A negative signal was shown for **slots 6 and 7** which corresponds to 10^{-1} and 10^{-2} pfu, respectively, of Coxsackievirus B3 per milligram of cardiac tissue. (T+, positive control of amplification; T−, negative control of amplification.

DISCUSSION

The persistence of enteroviral RNA in myocardium from patients with DCM has been reported, but the prevalence of enteroviral heart infection in chronic cardiomyopathies is subject to controversy [Petitjean et al., 1992; Giacca et al., 1994]. To study the prevalence of EV RNA detection in myocardium from patients suffering from chronic cardiomyopathy, we selected controls divided in two subgroups; one subgroup included 11 children, and the second included 21 adult organ donors. These two subgroups were free from any known heart disease, unlike the case in previous reports in which control groups included patients suffering from heart diseases other than DCM [Weiss et al., 1991; Petitjean et al., 1992; Keeling et al., 1992]. None of the endomyocardial biopsies from the control group was EV RNA positive. These data demonstrated that the negativity of the RT-PCR assay correlated well with the absence of cardiac histological abnormalities, which argues for the absence of asymptomatic acute or chronic EV heart infection in immunocompetent patients.

In the DCM group, the failure of detection by cell culture in RT-PCR-positive endomyocardial recipients demonstrated the value of the genomic amplification assay to detect low-grade viral infection in end-stage cardiac disease. A single subculture during 14 days carried out on a single cell type might not result in a positive cell culture detection as has been previously suggested [Chonmaitree et al., 1988]. The positive RT-PCR results (57.9%) in the DCM group were in agreement with those (66%) reported by Petitjean et al. [1992] but were higher than those (12%) observed by Keeling et al. [1992] or those (7.5%) found by Giacca et al. [1994]. However, the sequence of our primers was different from those used in previous studies, and an internal control was amplified to verify the sufficient transcription of cDNA and the removal of Taq-DNA polymerase inhibitors. EV RNA detection was positive in 80% of the patients suffering from idiopathic dilated cardiomyopathy, suggesting the possible role of enteroviruses in the pathogenesis of DCM (Table I). The failure to detect EV genomic sequences in only two patients with idiopathic DCM may be explained by the lack of sensitivity of amplified products detection. Another possibility for the negative RT-PCR results is that a single ventricular biopsy is not sufficient for detecting a multifocal, randomly distributed process such as EV myocardial infection [Kandolf et al., 1993].

We demonstrated that EV RNA sequences may be found in the endomyocardial tissue from several alcohol addicts suffering from DCM. The results must be considered with caution due to the low number of patients in this subgroup; however, alcohol seems to be the major etiology of DCM and the significance of EV detection in alcoholic cardiomyopathy remains unknown [Why et al., 1994]. Nevertheless, the positive results in the alcoholic and congenital cardiopathy subgroups indicated that a preexisting cardiomyopathy might be a predisposing factor for multifocal cardiac EV infection [Liljeqvist et al., 1993].

The percentage of patients showing EV RNA was similar between CCD and DCM groups, pointing to a link between chronic cardiac disease and an EV heart infection. Age and sex do not appear to be important

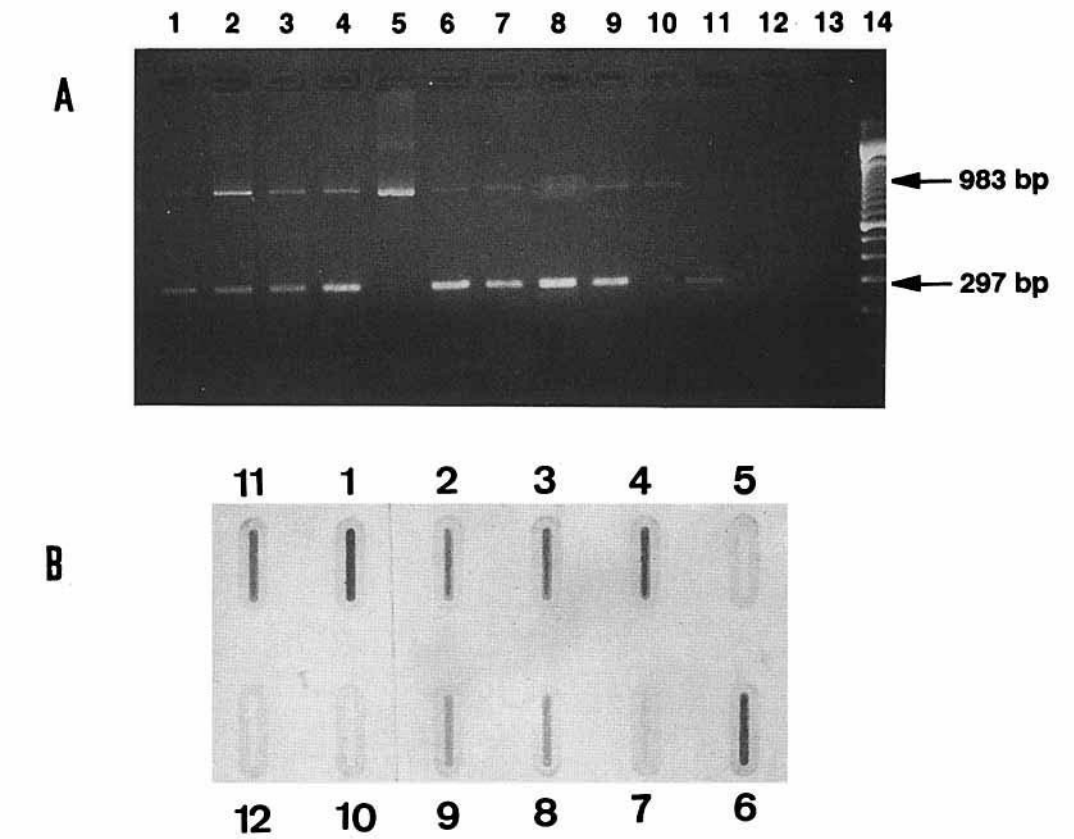

Fig. 2. **A:** Agarose gel electrophoresis revelation of RT-PCR products. This amplification was carried out in myocardium from patients suffering from DCM **(lanes 1–5)** and CCD **(lanes 6–10).** An enteroviral-specific fragment of 297 bp can be seen in lanes 1–4 and 6–9 but not in lanes 5 and 10. **Lane 11** corresponds to the positive control and **lane 12** to the negative control; **lane 13,** not used; **lane 14,** molecular weight markers (100 bp DNA ladder). The fragment of 983 bp corresponds to the amplified products of the G3PDH sequences and was used to validate the enteroviral RT-PCR assay for each specimen. **B:** Slot blotting revelation of enteroviral RT-PCR products presented in A. **Slots 1–5** correspond to patients with DCM and **slots 6–10** to patients with CCD. **Slot 11** is a positive enteroviral amplification control and **slot 12** a negative enteroviral amplification control. Positive signal is shown in slots 1–4 and 6–9.

factors affecting the EV prevalence in human heart tissue [Why et al., 1994]. Moreover, the absence of EV genomic detection in children and adult controls shows that this chronic heart infection is not a pathology that can occur asymptomatically during the life.

The implication of EVs as an enhancing factor in the pathogenic progress of chronic heart disease such as CCD should be considered. In previous studies, 17–39% of patients with CCD demonstrated the presence of EV RNA [Petitjean et al., 1992; Keeling et al., 1992]. The significance of EV infection in CCD patients is not known. EV may infect vascular endothelial cells in EV-infected mice, and myocarditis can be associated with microvascular spasm, small vessel obstruction, and myocardial reperfusion at the onset of infection [Sole and Liu, 1993]. Enteroviruses may be responsible for endothelial lesions enhancing vascularitis, artheromatosis, ischaemia, and myocardial infarction. These findings, together with the absence of EV RNA in myocardium from controls with myocardial infarction in the present study, support the involvement of enteroviruses in chronic ischaemic heart diseases.

Recent preliminary studies indicated that enteroviruses may persist in a defective mutant form, which may account for the lack of inflammatory response [Bowles et al., 1989; Cunningham et al., 1990]. In our study, the EV RNA detection during end-stage chronic cardiac diseases showed that, in spite of viral clearance by the immunological system, EV RNA single strands can persist for a long time in human cardiac tissue.

TABLE I. Detection of Enteroviral RT-PCR Results Among Patients Suffering From Chronic Cardiomyopathy or Myocardial Infarction and Healthy Heart Controls

Groups	Subgroups	Total (n)	RT-PCR		Percentage positive
			Positive	Negative	
DCM[a]	Alcoholic cardiopathy	7	1	6	14.3
	Idiopathic DCM	10	8	2	80
	Congenital cardiopathy	2	2	0	100
CCD[b]		14	8	6	57.1
DCM + CCD		33	19	14	57.6
Myocardial infarction[c]		33	0	33	0
Healthy heart controls[d]	Children	11	0	11	0
	Adult organ donors	24	0	24	0

[a]DCM, dilated cardiomyopathy. This group included patients with chronic dilated cardiomyopathy who required heart transplantation.
[b]CCD, chronic coronary disease. This group included patients with chronic ischaemic cardiomyopathy who required heart transplantation.
[c]The myocardial infarction, group of patients were suffering from endomyocardial infarction treated by coronary bypass.
[d]The healthy heart controls, group of subjects included children and adult organ donors without any cardiac disease.

This EV genomic persistence strongly suggests the existence of negative strands of EV RNA utilized as a template to synthetize new positive strands. Enteroviral persistence can be explained by abnormal production of equal amounts of positive and negative strands of RNA [Cunningham et al., 1990]. The excess amount of minus strands of RNA could imply a decreased capsid protein synthetisis, which could explain enteroviral evasion of the host immunologic surveillance system [Oldstone, 1989; Klingel et al., 1992].

In conclusion, the results of this study suggest that the pathogenesis of DCM and CCD is associated with EV infection and that these viruses may enhance the pathogenic process of CCD and DCM. The involvement of the EV group in chronic cardiomyopathy remains an intriguing question and further studies are needed to define the precise role of these viruses in the pathogenesis of chronic cardiac diseases.

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